

Generation of Adenosine Triphosphate in Cytochrome-deficient Mutants of *Neurospora**

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SUMMARY

The fungus *Neurospora crassa* is known to possess a branched respiratory system consisting of the standard cytochrome chain and a cyanide-insensitive alternate oxidase. In the present experiments, the physiological function of the alternate oxidase has been analyzed by taking advantage of a number of cytochrome-deficient mutants, particularly *poky f*. Respiration, cellular ATP levels, and growth have been examined under the influence of three classes of inhibitors: inhibitors of the cytochrome chain (antimycin, cyanide), an inhibitor of the alternate oxidase (salicyl hydroxamic acid), and an uncoupling agent (carbonyl cyanide *m*-chlorophenylhydrazone). The results indicate that the over-all efficiency of the alternate oxidase in producing ATP and supporting growth is much less than that of the cytochrome chain. Depending upon the amount of oxidative phosphorylation at Sites II and III in the cytochrome chain, which varies from strain to strain, the efficiency of the alternate oxidase relative to that of the cytochrome chain ranges from 13% in wild type *Neurospora* to 18 to 21% in *poky f*, 35% in *mi-3*, and 57% in *cyt-2*.

A comparison of the short term effects of cyanide and carbonyl cyanide *m*-chlorophenylhydrazone on cellular ATP in *poky f* suggests that, during respiration through the alternate oxidase, ATP can be produced both by substrate-level phosphorylation (accompanying glycolysis and the oxidation of α -ketoglutarate) and by oxidative phosphorylation at Site I. When cells are grown on sucrose, as much as 22% of ATP synthesis in the presence of cyanide occurs at Site I. When cells are grown on acetate to diminish the rate of glycolysis, the contribution of Site I becomes proportionately larger.

Both the growth experiments and the short term inhibitor experiments reveal that ATP levels in *Neurospora* are kept high by a feedback process which depresses ATP breakdown (and growth) very quickly after ATP synthesis is inhibited. Thus, *poky f* grows more slowly than wild type *Neurospora* and is inhibited still further when either the cytochrome

chain or the alternate oxidase is blocked. Under all of these conditions, however, cellular ATP in *poky f* is maintained at a high level (about 3 mmol per kg of cell water, slightly above the values measured in the wild type strain). The feedback process can be observed directly in short term experiments, where inhibition of the cytochrome chain by cyanide causes an initial sharp decline of cellular ATP to about 40% of the control value, followed by a recovery (perhaps with oscillations) to almost 80% in 2 min. Measurements of AMP and ADP, as well as ATP, make questionable the direct involvement of these adenine nucleotides in the feedback control, so that some other mediator (such as a cyclic nucleotide) should be sought.

Under most experimental conditions, respiration in wild type *Neurospora crassa* occurs by way of a standard cytochrome chain, consisting of two *b*-type cytochromes, cytochromes *c* and *c*₁, and cytochrome *aa*₃ (1-6). When the cytochrome chain is defective, however, in mutants such as *poky* (1-3, 5-7) or in wild type cells grown in the presence of antimycin, cyanide, or chloramphenicol (3, 7, 8), an alternate oxidase is induced. The alternate oxidase and the cytochrome chain have been shown to compete for reducing equivalents on the oxygen side of the dehydrogenases, thus forming a branched electron transport system (2, 3, 5, 7); and the two branches can be assayed separately by their sensitivity to specific inhibitors (antimycin and cyanide in the case of the cytochrome system; salicyl hydroxamic acid in the case of the alternate oxidase (2, 3, 5, 7)).

The role of the alternate oxidase in ATP production is not yet clear. In mitochondria isolated from *poky* or from wild type grown in the presence of chloramphenicol, essentially all of the oxidative phosphorylation is inhibited by antimycin or cyanide, and presumably occurs at Sites II and III of the residual cytochrome chain (3). It is conceivable, of course, that there is additional oxidative phosphorylation *in vivo*, e.g. linked to the alternate oxidase at a labile Site I. In addition, the alternate oxidase may well stimulate substrate-level ATP production by permitting glycolysis and the Krebs cycle to operate at an increased rate.

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To investigate these possibilities, we have carried out a series of ATP measurements on whole cells of *poky* and wild type *Neurospora*. The experiments were designed to establish the steady state levels of ATP in exponentially growing cells and the sensitivity of the ATP levels to inhibitors of both branches of the respiratory system.

METHODS

Strains of *Neurospora*—Most of the work described in this paper was carried out with wild type strain RL21a and with *poky* strain NSX f a, supplied by Rifkin and Luck and derived from a cross of *poky* f (3627-1) with *chol*-1 (34486) (Ref. 9). f was originally isolated as a nuclear-gene suppressor of the *poky* mutation, increasing the growth rate of strains which carry it without restoring the wild type respiratory system (10).

Additional strains were *mi*-3 (DR 253A, 256a, 270a) and *mi*-3 f (DR 275A, 276A, 279A) from a cross of *mi*-3 a (3754; FGSC No. 382) with *arg*-5 f A (from NSX f a \times 27947A); *cyt*-2 (C117a; FGSC No. 1981); and f (DR 227A), from a cross of RL21a with *arg*-5 f A. In the case of *mi*-3 f and f, the presence of the f allele was confirmed by crossing with *poky*.

Growth of Cells—Liquid cultures were prepared by inoculating conidia at a density of 10^6 per ml into standard minimal medium (Vogel, Ref. 11). In most cases, 2% sucrose served as a carbon source; where indicated, it was replaced by 0.5% acetate (1.13% sodium acetate/3 H₂O; pH 5.8). The cultures were grown at 25° either in 1-liter Florence flasks (160 ml of medium) on a reciprocating shaker (140 strokes per min), or in 3- or 6-liter flasks (1.5 or 3 liters of medium) vigorously bubbled with air.

O₂ Consumption—O₂ consumption was measured with a Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio). Cells were suspended in aerated growth medium in a glass reaction chamber and maintained at 25°. At the end of each measurement, the cells were harvested quantitatively from the chamber, rinsed, dried, and weighed. Results are expressed as microliters of O₂ consumed per mg dry weight per hour.

Measurement of Adenine Nucleotides—The assays for cellular ATP, ADP, and AMP have been described previously (12). In the present experiments, cells were harvested from mid-exponential phase cultures (15 to 16 hours for wild type, 21 hours for *poky* f), rinsed with distilled water, and resuspended at a density of 1 to 2 mg dry weight per ml in standard buffer (20 mM 3,3-dimethylglutaric acid, brought to pH 5.8 with KOH (final concentration 25 mM), 1 mM CaCl₂, and 1% glucose). (In a few instances, the cells were assayed directly in the growth medium.) The cell suspension was kept aerated by shaking and at measured intervals during growth or after injection of the inhibitor the suspension was poured through a sintered glass filter and the resulting cell pellet dashed into petroleum ether-Dry Ice. Each frozen pellet was lyophilized, weighed, and extracted into perchloric acid (12). The extracts were assayed for ATP by the firefly luciferase method of Strehler (13), and for ADP by the same method, after conversion to ATP with phosphocreatine plus creatine kinase. AMP was assayed fluorometrically using the pyruvate kinase-lactic dehydrogenase system (12). For comparison with previously reported values, results are expressed as millimoles of ATP, ADP, or AMP per kg of cell water, even though it is recognized that cellular nucleotides may not be distributed uniformly. Calculations of intracellular concentration were made from the previously determined value (2.54) for the ratio of intracellular water to dry weight, based on inulin and mannitol estimates of the extracellular space (14).

Reagents—Antimycin (Sigma), salicyl hydroxamic acid (Aldrich), and carbonyl cyanide *m*-chlorophenylhydrazine (Sigma) were dissolved in absolute ethanol; KCN (Baker) was dissolved in double-distilled water and neutralized. All of the inhibitors were prepared fresh for each experiment.

Computations—Data are presented throughout as mean values for the listed number of observations, with scatter indicated as ± 1 S.E.M. In a number of cases, the data were fitted to specific functions (see Figs. 5, 7, and 11, and Tables II and III) with the aid of a generalized nonlinear curve-fitting program (15), which was run on the Yale Computer Center's IBM 370 computer. Parameter estimates from these fits are bracketed by ± 1 S.E.

RESULTS

Respiration and ATP during Exponential Growth

Our initial objective was to compare respiration rates and cellular ATP levels in exponentially growing cultures of *poky* f and wild type. Previous work had shown that, in the standard minimal medium (Vogel's plus 2% sucrose), wild type strain RL21a grows with a doubling time of 2.5 hours at 25° (14). Virtually all of its respiration proceeds by way of the cytochrome chain; the respiration rate declines slowly from 90 to 60 μ l per mg dry weight per hour during the exponential phase, but at each point it is 90 to 95% sensitive to cyanide (1 mM) and less than 5% inhibited by salicyl hydroxamic acid (1 mM) (7). Cellular ATP rises gradually and then falls again, with peak values of 2.1 to 2.3 mmol per kg of cell water during the mid-log phase, at 12 to 14 hours (12).

The corresponding functions for *poky* f are shown in Fig. 1. Growth is slower, with a doubling time of 4.5 hours (see also Ref. 7). During the exponential phase, from 6 to 22 hours, the respiration rate of *poky* f is extremely high, ranging from 160 to 210 μ l per mg dry weight per hour. Previous work has shown that the over-all rate in *poky* f includes contributions from both branches of the respiratory system, with the cytochrome chain operating at maximal activity and the alternate oxidase used to accommodate surplus electron flow (2, 3, 7, 8). In any given situation, the activity of the cytochrome chain can be estimated as the fraction of respiration that is resistant to a saturating concentration of salicyl hydroxamic acid (1 mM; Ref. 7), and amounts to about 50 μ l per mg dry weight per hour or one-

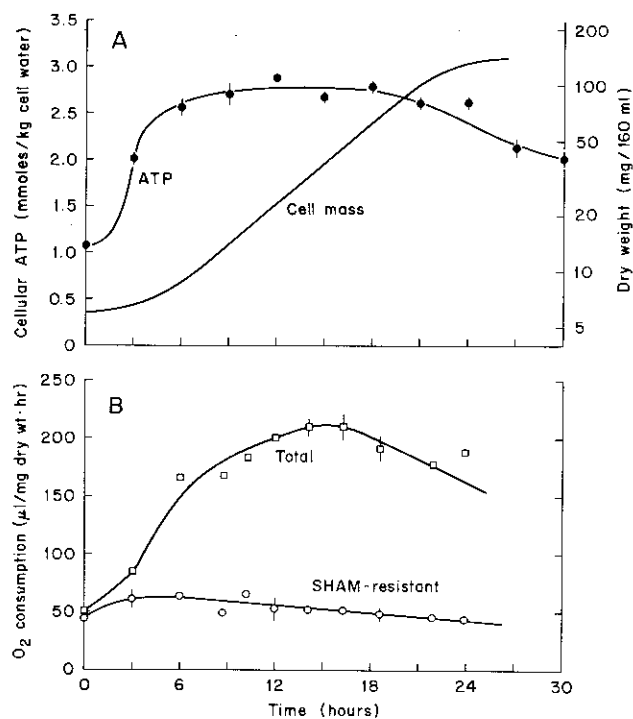


FIG. 1. Cellular ATP levels (A) and respiration rates (B) in the *poky* f strain of *Neurospora crassa* during exponential growth in Vogel's minimal medium containing 2% sucrose. Curve of cell mass is redrawn from Lambowitz and Slayman (7). Respiration rates were measured before and after adding salicyl hydroxamic acid (SHAM) (1 mM) to block the alternate oxidase; SHAM-resistant respiration represents the activity of the cytochrome chain (see text). Values plotted are averages for three separate determinations. Vertical bars, ± 1 S.E.M.

quarter of the total in the present experiment (Fig. 1B). The alternate oxidase accounts for the remainder (although this is not its maximal activity; addition of cyanide or antimycin to inhibit the cytochrome chain results in a shift of electron flow to the alternate oxidase, with very little effect on total respiration; Refs. 2, 3, 7; see also *inset* to Fig. 6, below).

Somewhat surprisingly, cellular ATP levels in exponentially growing *poky f* are consistently higher than those in wild type *Neurospora*, ranging from 2.6 to 2.9 mmol per kg of cell water (Fig. 1A). This result suggests that ATP levels *per se* do not operate to control the growth rate in *Neurospora*, a point which will be discussed further below.

(Fig. 1 also contains information about respiration and ATP levels in conidia and during early germination, stages of growth which, although not a major focus of the present work, are of interest in themselves. Like wild type conidia (12), *poky f* conidia respire slowly (at about 50 μ l per mg dry weight per hour) and contain relatively little ATP (1.1 mmol per kg of cell water). Both of these functions rise dramatically during germination. A special feature of *poky f* is that the rise in respiration involves the alternate oxidase almost exclusively (Fig. 1B) and the cytochrome chain continues to operate at the same slow rate during the entire exponential phase. This result implies that respiration through the alternate oxidase can stimulate the production of ATP, as is discussed in greater detail below.)

In order to determine the relative roles of the cytochrome chain and the alternate oxidase in supporting growth, an experiment was next carried out in which specific inhibitors of the two pathways (antimycin, 1 μ g per ml; salicyl hydroxamic acid, 1 mM) were added to the growth medium at the mid-log phase (12 hours). Aliquots were withdrawn during the following 4 hours for the measurement of cell mass, ATP content, and respiration. (For this experiment, antimycin was used as an inhibitor of the cytochrome chain because cyanide is volatile and its concentration in the growth medium would decrease substantially over a 4-hour period. Previous work had shown that neither antimycin nor cyanide blocks respiration through the alternate oxidase (2, 7).)

In the wild type strain, as expected, the addition of antimycin to the medium inhibited respiration by more than 90% (Fig. 2A) leading to a sharp drop in cellular ATP (Fig. 2D) and complete cessation of growth (Fig. 2C). By contrast, salicyl hydroxamic acid depressed respiration slightly (by about 20%) but had no detectable effect on ATP and very little effect on growth (the doubling time increased from 2.9 hours in the control to 3.6 hours in the presence of salicyl hydroxamic acid). These results are consistent with the idea that the cytochrome system plays by far the dominant role in normal exponential growth of

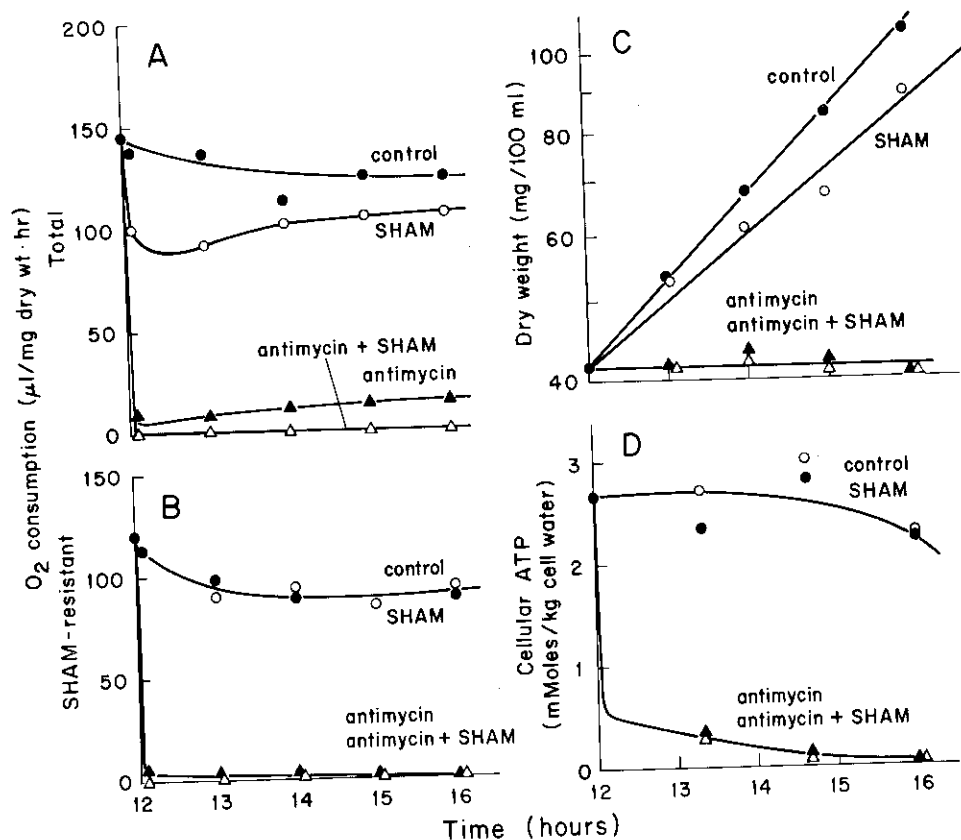


FIG. 2. Effects of antimycin and salicyl hydroxamic acid (SHAM) on growth (C), respiration (A, B), and cellular ATP (D) in wild type *Neurospora*. Cells were grown in 1.5-liter bubbled cultures in Vogel's medium plus 2% sucrose under the standard conditions (except that the inoculum was 2 to 4×10^6 conidia per ml) for 12 hours before the addition of inhibitors. Cell dry mass was determined on pellets harvested and rinsed from 100-ml aliquots. Doubling times, computed by linear least squares fitting on semilog plots (C), were: control, 2.80 ± 0.05 hours; SHAM,

3.74 ± 0.17 hours. ATP and respiration were determined on 25-ml aliquots which were removed 20 to 30 min prior to the assay time and incubated (still in the original growth medium) on a reciprocating shaker. SHAM-resistant respiration (B) was measured by the addition of SHAM (1 mM) to cells in the respiration chamber, after the total respiration rate (A) had been established (in the presence of antimycin, SHAM, both, or neither from the growth medium). Values for ATP content are averages of three measurements; all other values are averages of two measurements.

wild type *Neurospora*. When the cytochrome system is blocked, ATP levels cannot be maintained and growth cannot occur.

In *poky f*, on the other hand, either the residual cytochrome chain or the alternate oxidase proved capable of supporting growth, although at quite different rates. When antimycin was added to the growth medium, respiration shifted completely to the alternate oxidase (total respiration rose slightly above the control value, but was 98% blocked by salicyl hydroxamic acid; Fig. 3, A and B). The control ATP level was maintained (Fig. 3D), but exponential growth was slowed by 44% (doubling time of 7.7 hours, compared with 4.3 hours in the control; Fig. 3C). In a parallel experiment, when salicyl hydroxamic acid was added to the growth medium, respiration was initially restricted to the cytochrome chain. Total respiration was reduced from the control value of 208 μ l per mg dry weight per hour to about 65 μ l per mg dry weight per hour (Fig. 3A) and was resistant to salicyl hydroxamic acid (Fig. 3B) but sensitive to cyanide (data not shown). Again, the cellular ATP level was not significantly changed, but growth was slowed by 16% (doubling time was 5.1 hours). (The effect of salicyl hydroxamic acid appeared to "wear off" with time, so that by 15 hours, respiration had risen to 123 μ l per mg dry weight per hr; the readdition of salicyl hydroxamic acid brought respiration back to the initial inhibited value (Fig. 3B).) Finally, antimycin + salicyl hydroxamic acid, as expected, blocked respiration completely and caused cellular ATP to fall and growth to stop.

A more quantitative interpretation of these results can be gained by assuming that the growth rate is proportional to the

activity of the cytochrome chain plus some smaller fraction of the activity of the alternate oxidase:

$$\frac{d \text{ Mass}}{dt} \sim \frac{1}{\text{doubling time}} = \alpha \left[\left(\frac{dO_2}{dt} \right)_{\text{cyt}} + \beta \left(\frac{dO_2}{dt} \right)_{\text{alt}} \right] \quad (1)$$

The four cases which give usable data for this equation are listed in Table I. (Because respiration and ATP tended to change somewhat with time, the values in Table I are arbitrarily taken from the curves of Fig. 3 at 14 hours, the middle of the interval of study.) Fitting the data to Equation 1, with a least squares program for the sum of two straight lines (15), yields the following parameters: $\alpha = 0.00253$ and $\beta = 0.210$. In other words, in *poky f*, the alternate oxidase appears to be about 21% as efficient as the cytochrome chain in supporting growth.

Growth of Other Cytochrome-deficient Mutants

A second approach toward evaluating the efficiencies of the two pathways makes use of a series of *Neurospora* mutants with quantitatively different defects in the activity of the cytochrome chain. In the present experiments, strains included *poky*, *poky f* (where *f*, a nuclear-gene suppressor, increases the growth rate without restoring the wild type cytochrome system; Refs. 1 and 10), *mi-3* (an independently isolated cytoplasmically inherited mutant with reduced amounts of cytochrome *aa₃* but normal amounts of cytochromes *b* and *c*; Refs. 5 and 16), *mi-3 f*, and, as controls, the wild type strain and the single mutant *f*. In each case, measurements were made of the exponential growth rate, total respiration, and respiration resistant to salicyl hy-

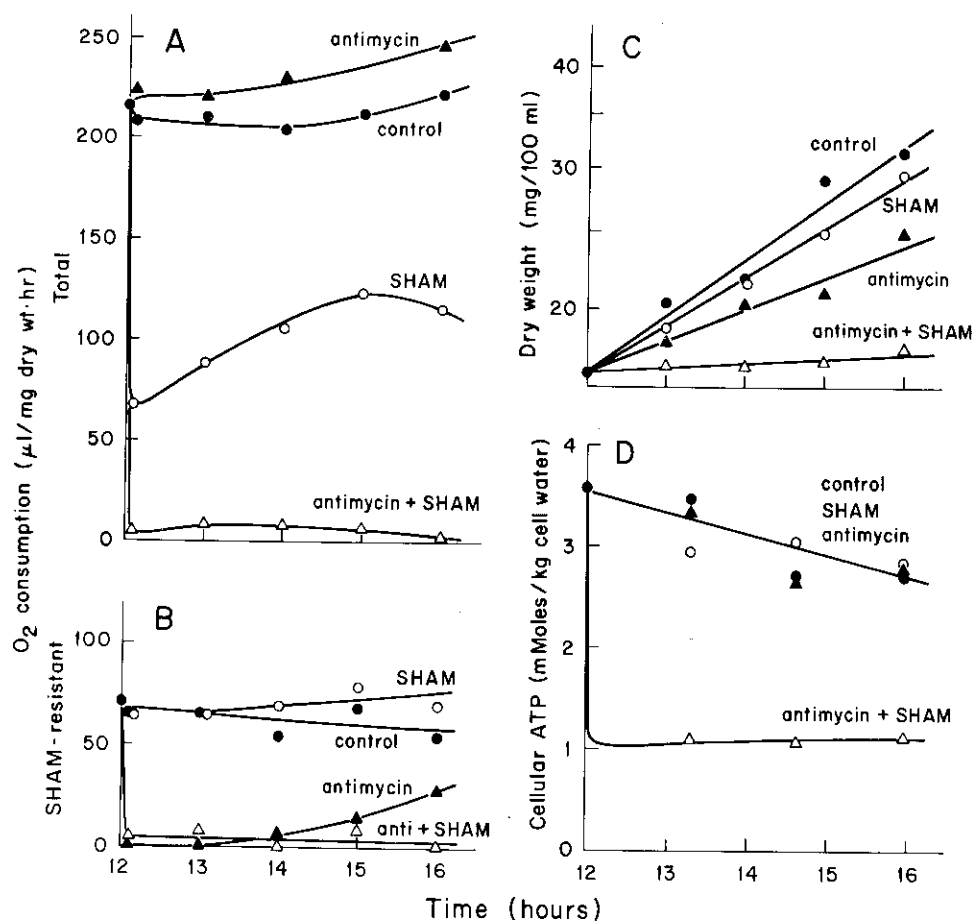


FIG. 3. Effects of antimycin and salicyl hydroxamic acid (SHAM) on growth (C), respiration (A, B), and cellular ATP (D) in *poky f*. Protocol exactly the same as in Fig. 2. Doubling times for growth are listed in Table I.

droxamic acid (the latter two quantities showing relatively little variation over the period 10 to 22 hours). The data are listed in Table II; and in Fig. 4, the growth rate is plotted as a function of both types of respiration. For all six strains, growth appeared to be directly proportional to salicyl hydroxamic acid-resistant respiration (*i.e.* to the activity of the cytochrome chain) but roughly inversely proportional to the total respiration.

Again, as in the previous experiment, the results were fitted to Equation 1, yielding values of 0.0041 for α and 0.0379 for β . From this set of measurements, therefore, the alternate oxidase appears at first sight only 3.8% as efficient as the cytochrome chain in supporting growth, a value substantially lower than the one obtained from inhibitor measurements on *poky f* (21%; see "Effects of Respiratory Inhibitors on Growth"). The discrepancy

probably arises from incorporating into Equation 1 the assumption that the efficiency of cytochrome-linked phosphorylation, as distinct from the fraction of cytochrome chain which may be present, is strain-independent. In fact, data from isolated mitochondria make this assumption quite unlikely. Lambowitz *et al.* (3) found phosphorus to oxygen ratios of 1.3 for wild type mitochondria respiring on succinate, and phosphorus to oxygen ratios around 0.75 for *poky f* mitochondria respiring on succinate in the presence of salicyl hydroxamic acid. Therefore, in order to reformulate the equation relating growth rate to respiration, and in the absence of specific data on the phosphorylating efficiency at Sites II and III in the several strains shown in Fig. 4, we have made the arbitrary assumption that the efficiency at Sites II and III is linearly proportional to the amount of cytochrome respiration in uninhibited cells. Equation 1 is then transformed into

$$\frac{1}{\text{doubling time}} = \alpha \left[\frac{1-\beta_1}{\gamma} \left(\frac{dO_2}{dt} \right)_{\text{cyt}}^2 + \beta_1 \left\{ \left(\frac{dO_2}{dt} \right)_{\text{cyt}} + \left(\frac{dO_2}{dt} \right)_{\text{alt}} \right\} \right] \quad (2)$$

TABLE I
Growth and respiration of *poky f* in presence of inhibitors
Results tabulated from Fig. 3. Doubling times (± 1 S.E.) were calculated from the least squares slopes of the lines in Fig. 3C. Rates of respiration are the values at 14 hours in Fig. 3, A and B. Respiration via the cytochrome chain is that remaining in the presence of 1 mM salicyl hydroxamic acid; respiration via the alternate oxidase is the difference between total respiration and cytochrome respiration (see text).

	Doubling time	1/Doubling time	Respiration		
			Cytochrome chain	Alternate oxidase	Total
	hr	hr ⁻¹	$\mu\text{g/mg dry wt/hr}$		
Control	4.26 \pm 0.22	0.235	63	143	206
SHAM ^a	5.10 \pm 0.11	0.196	69	40	109
Antimycin	7.66 \pm 0.44	0.131	5	222	227
Antimycin + SHAM ^a	53.8 \pm 5.8	0.0186	3	5	8

^a SHAM is salicyl hydroxamic acid.

Here $(dO_2/dt)_{\text{cyt}}/\gamma$ is the proportionality coefficient relating the efficiency at Sites II and III to the amount of cytochrome respiration; if this quantity is defined as 1.0 for wild type *Neurospora*, then $\gamma = (dO_2/dt)_{\text{cyt(wild type)}}$. β_1 is the combined efficiency of substrate-level phosphorylation and oxidative phosphorylation at Site I (*i.e.* all of the phosphorylation that takes place prior to the branch point between the cytochrome chain and the alternate oxidase; Refs. 2-5); for the sake of simplicity, we have assumed that these processes are independent of the amount of cytochrome respiration (*i.e.* β_1 is constant). When Equation 2 is fitted separately to the data in Table I and in Fig. 4, the values of β_1 obtained are 0.15 and 0.16, respectively. These values are essentially identical and imply that phosphorylation supported by the alternate oxidase is 15 to 16% as efficient as phosphorylation via wild type Sites II and III. Various parameters of the fits, plus observed and predicted values of the re-

TABLE II
Relationship of growth rate to respiration via cytochrome chain and alternate oxidase in seven strains of *Neurospora*

Cells were grown in 3-liter bubbled cultures, and cell mass and respiration were determined at intervals from 8 to 16 hours for the wild type and from 8 to 24 hours for the mutants. Doubling times were computed by linear least squares from semilog plots of cell mass *versus* time. Values for respiration are averaged over the whole sampling period. Results for *f* are from a single ex-

periment; all of the others are averages of two or three experiments. The Marquardt algorithm (15) was used to fit both Equation 1 and Equation 2 (see text) to the data. The value of $\gamma = 96.1$ was assigned, as the average value of cytochrome respiration in wild type cells.

Strain	Respiration		Doubling time	1/Doubling time	1/Doubling time (predicted)	
	Total	SHAM ^a -resistant			Eq. 1	Eq. 2
	$\mu\text{g/mg dry wt/hr}$		hr		hr ⁻¹	
wild type	105.5 \pm 19.4	96.1 \pm 15.9	2.46 \pm 0.03	0.407	0.394	0.400
<i>f</i>	109.3	96.5	2.56	0.391	0.397	0.405
<i>mi-3 f</i>	168.5 \pm 9.1	59.7 \pm 1.6	3.83 \pm 0.16	0.261	0.261	0.239
<i>mi-3</i>	168.5 \pm 10.5	51.2 \pm 2.8	4.16 \pm 0.13	0.240	0.228	0.206
<i>poky f</i>	201.4 \pm 7.2	55.8 \pm 4.1	4.51 \pm 0.15	0.222	0.251	0.245
<i>poky</i>	207.5 \pm 17.5	34.2 \pm 1.8	5.90 \pm 0.20	0.169	0.167	0.179
<i>cyt-2</i>	182.9 \pm 2.1	22.0 \pm 3.2	7.73 \pm 1.19	0.129	0.115	0.139
Fitting parameters						
α					0.00409 \pm 0.00012	0.00411 \pm 0.00017
$\beta(\beta_1)$					0.0379 \pm 0.0161	0.161 \pm 0.020
γ						= 96.1
Σd^2					1.4 $\times 10^{-3}$	2.6 $\times 10^{-3}$

^a SHAM is salicyl hydroxamic acid.

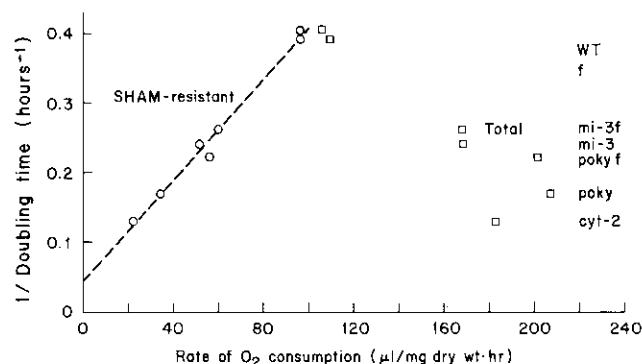


FIG. 4. Growth of wild type *Neurospora* and several cytochrome-deficient mutants, plotted as functions of total respiration and salicyl hydroxamic acid (SHAM)-resistant (cytochrome) respiration. Protocol for the experiment is described in the legend to Table II.

reciprocal doubling times for the two cases, are listed in Tables II and III. It is obvious from the inspection of these results, particularly the sum of squared deviations (Σd^2), that Equation 1 gives at least as good a fit to the data as does Equation 2. The advantage of the latter equation is strictly that it yields equivalent values of β_1 for the two sets of data.

With a specific value of β_1 , say 0.15 for *poky f*, one can now calculate the over-all efficiency of the alternate oxidase relative to the cytochrome chain in *poky f* = $0.15/(0.15 + 0.69) = 0.18$. In this calculation, 0.15 can be put in both the numerator and denominator as long as no phosphorylation occurs along the pathway to the alternate oxidase after the branch between the cytochromes and the alternate oxidase. Here, 0.69 is the assumed phosphorylation efficiency of Sites II and III in *poky f* relative to that in wild type *Neurospora* ($0.69 = (dO_2/dt)_{\text{cyt}(poky f)} / (dO_2/dt)_{\text{cyt}(wild type)}$; see Table III). The result, 0.18, agrees well with that obtained directly from Equation 1 ($\beta = 0.21$); the deviation between the two values reflects an inverse deviation between the values of the parameter α (Table III). In the same way, one can also calculate the over-all efficiency of the alternate oxidase relative to a normal cytochrome chain = $0.15/(0.15 + 1.0) = 0.13$.

Short Term Effects of Inhibitors on Cellular ATP

From the results so far, we conclude that both the cytochrome chain and the alternate oxidase can contribute to the generation of ATP, and that whichever pathway supplies ATP (depending upon the strain and the presence of particular inhibitors), the rate of growth is somehow regulated to keep the cellular ATP level high (around 2.5 to 3.0 mmol per kg of cell water). This latter conclusion means that changes in the rate of ATP synthesis, as produced by adding antimycin or salicyl hydroxamic acid to the growth medium in the experiments of Fig. 3, must feed back to produce corresponding changes in the overall rate constant for ATP hydrolysis.

In order to pursue these points in greater detail, we carried out a series of experiments to examine the short term effects of cyanide and salicyl hydroxamic acid on cellular ATP in *poky f* and wild type. The experiments were designed to give further information about sites of ATP synthesis linked to the cytochrome chain and the alternate oxidase and also to see whether the feedback phenomenon could be observed directly. For this portion of the work, cells were removed from the growth medium and resuspended in a standard buffer (20 mM dimethyl-

TABLE III

Relationship of growth rate to respiration via cytochrome chain and alternate oxidase in *poky f*, growing in presence of inhibitors

The Marquardt algorithm (15) was used to fit Equations 1 and 2 (see text) to the data from Fig. 3. The value of $(dO_2/dt)_{\text{cyt}}/\gamma = 0.687$ was assigned, as the average of cytochrome respiration in *poky f* (66 μl per mg dry wt per hr; Table I) divided by that in wild type *Neurospora* (96 μl per mg dry wt per hr; Table II).

	1/Doubling time		
	Observed	Predicted (Eq 1)	Predicted (Eq 2)
Control	0.235	0.235	0.235
SHAM ^a	0.196	0.196	0.196
Antimycin	0.131	0.130	0.130
Antimycin + SHAM ^a	0.0186	0.0102	0.0102
Fitting parameters			
α		0.00253 ± 0.0007	0.00345 ± 0.00011
$\beta(\beta_1)$		0.210 ± 0.014	0.154 ± 0.011
$dO_2/dt_{\text{cyt}}/\gamma$			$= 0.687$
Σd^2		7×10^{-5}	7×10^{-5}

^a SHAM is salicyl hydroxamic acid.

glutarate, 1 mM CaCl_2 , 1% glucose, pH 5.8). Dimethylglutarate buffer has been used for a large number of related experiments (measurements of ion transport (17) and membrane potential (18)) and a previous study has shown that wild type cells, resuspended in it, undergo a rise and stabilization of cellular ATP at 2.6 mmol per kg of cell water (12). In the present experiments, cellular ATP also rose in *poky f* until, after 20 min in the buffer, it averaged 3.4 ± 0.2 mmol per kg of cell water (18 determinations). Because of the difference in control ATP levels between *poky f* and wild type, inhibition curves have been plotted as a per cent of the control; actual values for each experiment are included in the legends.

Cyanide Plus Salicyl Hydroxamic Acid—The first step was to determine the response of cellular ATP to complete respiratory inhibition, brought about by the simultaneous addition of 1 mM cyanide plus 1 mM salicyl hydroxamic acid (Fig. 5). For both the wild type and *poky f*, the decline of ATP followed a simple exponential curve; the time constants were 6.4 s and 14.1 s, respectively. The total loss of ATP was 2.59 mmol per kg of cell water (= $2.84 - 0.25$) in the wild type and 3.14 mmol per kg of cell water (= $3.66 - 0.52$) in *poky f*. The apparent rates of ATP turnover, then, were 0.40 mm per s and 0.22 mm per s in the two cases, and it is interesting to note that the ratio of these rates (1.81) is almost identical with the ratio of exponential growth rates in the two strains (= 4.5:2.5; see previous section). It must be emphasized that 0.40 mm per s and 0.22 mm per s are apparent turnover rates which may be low by a factor of 3 to 4 because of transphosphorylation from other nucleoside di- and triphosphates (12).

As was observed in the growth experiments (Figs. 2D and 3D), cyanide + salicyl hydroxamic acid did leave a finite residuum of ATP in both wild type *Neurospora* and *poky f*: 8.8% and 14.2%, respectively, in the present case. Presumably, this ATP was synthesized by ethanolic fermentation (19, 20), but the residual fractions give only a gross upper limit estimate to the amount of fermentation occurring during normal respiration. Two other factors almost certainly play a large role, though

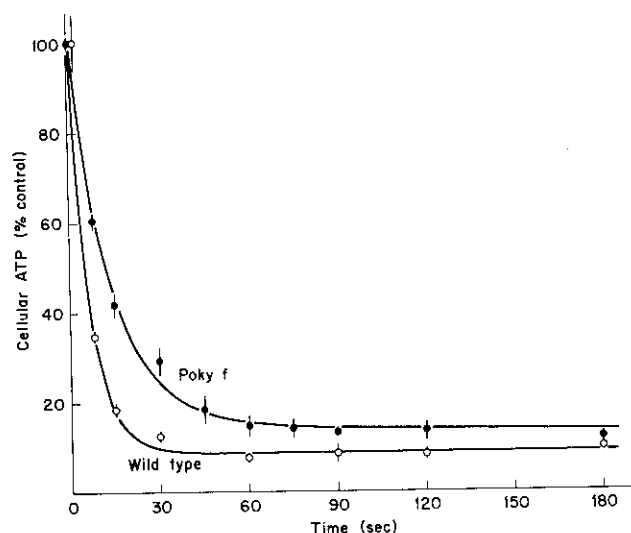


FIG. 5. Combined effect of cyanide plus salicyl hydroxamic acid, on cellular ATP in *poky f* and wild type *Neurospora*. Cells were harvested from exponential growth in Vogel's medium plus 2% sucrose at 15 to 16 hours (wild type) or 21 hours (*poky f*) and resuspended in the standard dimethylglutarate buffer for 20 to 90 min before the addition of inhibitors (at zero time). Each point is the average of three determinations; vertical bars, ± 1 S.E.M. Both curves were fitted to the equation $y = A_1 + A_2 e^{-t/\tau}$, with the values below obtained by least squares (15). Control ATP levels: 2.84 ± 0.18 mmol per kg of cell water for wild type; 3.66 ± 0.23 mmol per kg of cell water for *poky f*.

	A_1	A_2	τ
	%		s
Wild type	8.8 ± 0.6	91.2 ± 1.4	6.43 ± 0.24
<i>poky f</i>	14.2 ± 1.2	85.7 ± 2.5	14.1 ± 1.0

quantitatively undetermined, in sustaining finite ATP levels with complete respiratory blockade: Pasteur-effect release of fermentation, and the same feedback inhibition of ATP hydrolysis which was postulated above to account for the growth results. This problem is discussed further below.

Cyanide—With both strains, cyanide alone produced a more complex response of ATP than did cyanide + salicyl hydroxamic acid (Fig. 6). In the wild type, as described previously (18), ATP declined exponentially, with an average time constant of 5.7 s, to about 20% of the control, and then recovered to 25% in 3 min. The result is consistent with the idea that the bulk of ATP synthesis in wild type *Neurospora* depends upon respiration through the cytochrome chain.

In *poky f*, ATP declined less sharply to 43% in 20 s and then rebounded to 77% of the control value, at the same time showing a tendency to oscillate. (The existence of true oscillations in this rebound process is supported by the fact that the membrane potentials of individual hyphae from *poky f* display clear-cut, damped oscillations lasting 1 to 5 min after the addition of cyanide (21). Partial masking of the oscillations in the ATP measurement of Fig. 6 may have resulted both from scatter in the data and from an asynchronous summation of individual oscillations in the population of cells.) One can calculate, from the results reported in the previous section, the fractional ATP level which should persist in *poky f* when cytochrome respiration is blocked but the alternate oxidase continues to function. In the absence of feedback effects, this fraction equals $(1.0 \times 0.15) / (0.72 \times 0.15 + 0.28 (0.69 + 0.15)) = 0.44$, assuming the real

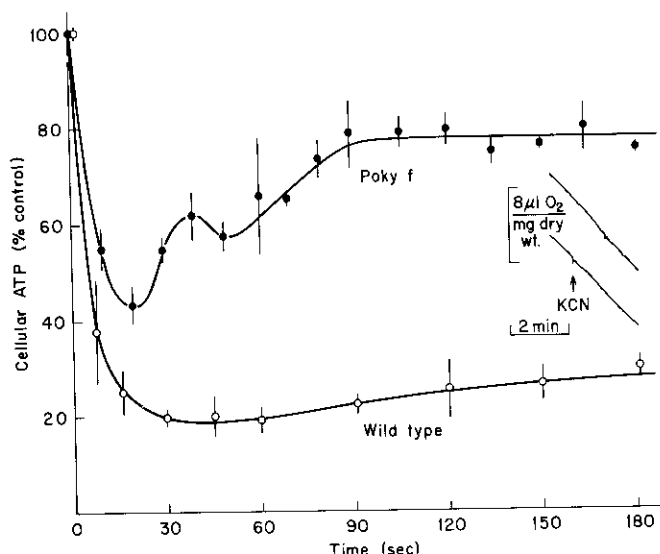


FIG. 6. Effect of cyanide (10^{-3} M) on cellular ATP in *poky f* and wild type *Neurospora*. Methods as in Fig. 5. Data for the wild type are replotted from Slayman (12); data for *poky f* were obtained in collaboration with Dr. Dietrich Gradmann, Yale University. Control ATP levels: 2.64 ± 0.06 mmol per kg of cell water for wild type; 3.45 ± 0.16 mmol per kg of cell water for *poky f*. Inset: two respiration traces for *poky f*, showing virtual lack of effect of cyanide (added at the arrow) on total respiration.

contribution from fermentation to be negligible, or it equals $0.86 \times 0.44 = 0.52$, assuming the contribution from fermentation to be maximal. In these equations, 0.86 is the minimal proportion of ATP dependent upon respiration (and 0.14 is the maximal proportion independent of respiration; see Fig. 5); 0.15 is the efficiency of phosphorylation supported by the alternate oxidase in *poky f* relative to phosphorylation at wild type Sites II and III; 0.69 is the phosphorylation efficiency of Sites II and III in *poky f* relative to wild type; and 0.28 is the fraction of total respiration in *poky f* which occurs via the cytochrome chain. (Average results from 17 respiration experiments with *poky f* gave a total respiration of $197.9 \pm 5.3 \mu\text{l}$ of O_2 per mg dry weight per hour and a salicyl hydroxamic acid-resistant (cytochrome) respiration of $55.2 \pm 2.1 \mu\text{l}$ of O_2 per mg dry weight per hour.) The calculation assumes that when cyanide is added, the alternate oxidase immediately accommodates the electron flow which spills over from the cytochrome chain, so that there is little if any change in the total respiration (inset to Fig. 6; see also Ref. 7). The immediate decline of ATP to 43% (Fig. 5) agrees very well with the first number calculated above, thereby strengthening the argument that ethanolic fermentation contributes little to ATP production during vigorous respiration. Again, it seems reasonable to conclude that the subsequent recovery of ATP to 77% of the control level arises from the same feedback mechanism which is involved in the regulation of growth.

Salicyl Hydroxamic Acid—A parallel analysis can be made for the action of salicyl hydroxamic acid on cellular ATP in *poky f*. In the absence of feedback effects, the fractional ATP level which should persist when the alternate oxidase is blocked (by salicyl hydroxamic acid) but the cytochrome chain continues to function equals $[0.28 (0.15 + 0.69)] / [0.28 (0.15 + 0.69) + 0.72 (0.15)] = 0.69 (0.73, \text{ if maximal fermentation is included})$, where 0.15, 0.69, and 0.28 are the values used in the previous calculation. In this case, because the cytochrome chain is already operating at maximal capacity in uninhibited cells, the addition

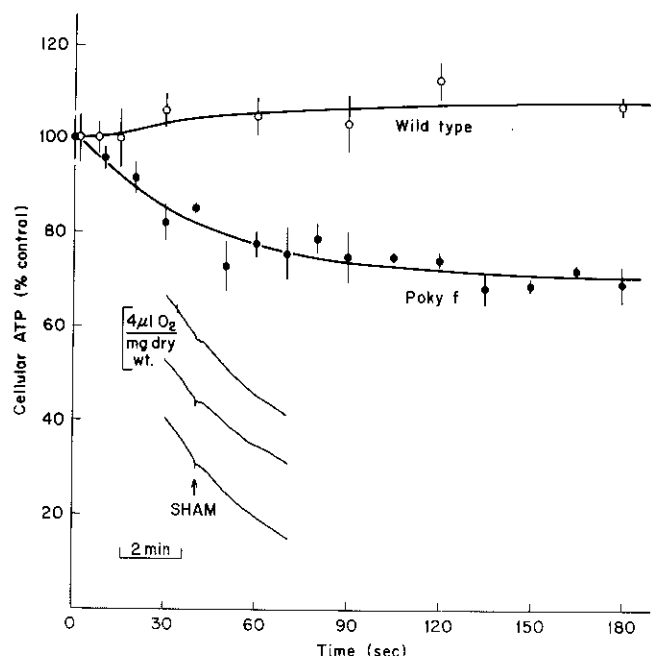


FIG. 7. Effect of salicyl hydroxamic acid (SHAM; 10^{-3} M) on cellular ATP in *poky f* and wild type *Neurospora*. Methods as in Fig. 5. The curve for *poky f* is drawn according to the equation $y = 69.7 \pm 30.3 e^{-t/41.8}$, with the numbers obtained as least squares estimates (15). Control ATP levels: 2.57 ± 0.13 mmol per kg of cell water for wild type; 4.11 ± 0.20 mmol per kg of cell water for *poky f*. Inset: three respiration traces for *poky f* showing the slow onset of SHAM inhibition.

of salicyl hydroxamic acid leads to a reduction of total respiration (inset to Fig. 7; see also Ref. 7).

The effects of salicyl hydroxamic acid (1 mM) on ATP are shown in Fig. 7. As expected, salicyl hydroxamic acid caused very little change in ATP levels in wild type *Neurospora*, producing, if anything, a slight increase (6 to 8%) during 3 min. In *poky f*, on the other hand, the same concentration of salicyl hydroxamic acid brought about an exponential decay of ATP, with a time constant of 42 s, to 70% of the control. This value agrees well with the predicted ones (69%, 73%) and the results show no evidence of a feedback effect during the 3-min period.

Carbonyl Cyanide *m*-Chlorophenylhydrazone—The short term inhibitor experiments described up to this point confirm the earlier conclusion that ATP synthesis is linked to respiration through the alternate oxidase, both during normal, uninhibited respiration of *poky f* and, even more visibly, during the recovery of cellular ATP that occurs in the presence of cyanide. In principle, this ATP synthesis might represent either substrate-level phosphorylation (accompanying glycolysis and the oxidation of α -ketoglutarate) or oxidative phosphorylation at Site I (which would be located just ahead of the branch point between the cytochrome chain and the alternate oxidase). In spite of the fact that the isolated mitochondria of *poky f* failed to yield evidence for Site I phosphorylation (3), the point seemed worth reinvestigating in intact cells.

The most direct approach to measure the contribution of Site I is to determine whether the recovery of ATP during exposure to cyanide is reduced or even eliminated altogether by the uncouplers of oxidative phosphorylation. One such uncoupler, known to be effective at low concentrations in isolated *Neurospora* mitochondria (3), is carbonyl cyanide *m*-chlorophenylhydrazone. In work with intact yeast cells, however, Kovac and Hrusovska (22) found that the related compound carbonyl cyanide *p*-tri-

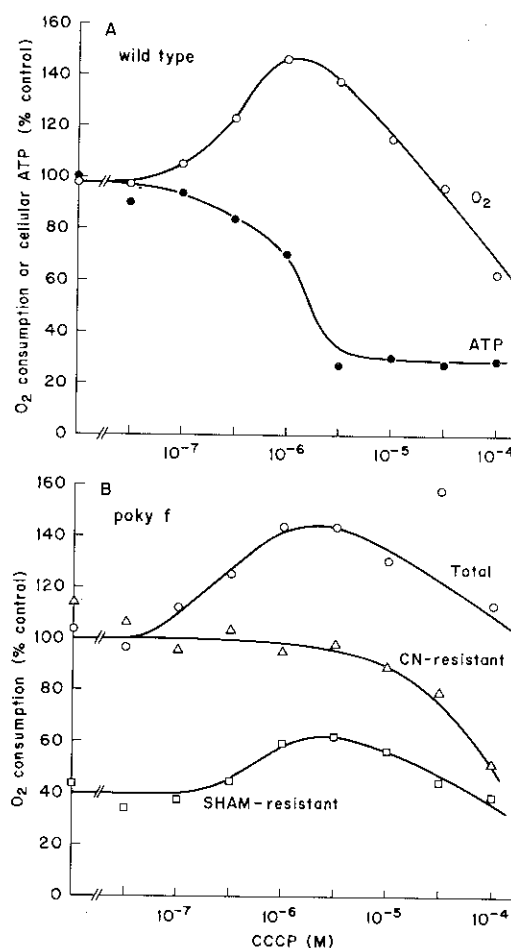


FIG. 8. Effects of different concentrations of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) on respiration and cellular ATP. A, wild type *Neurospora*. B, *poky f*. Cells were harvested from exponentially growing cultures at 15 to 16 hours (wild type) or 21 hours (*poky f*) and resuspended in the standard dimethylglutarate buffer for 40 to 90 min before use. CCCP was dissolved in ethanol, and all of the samples (including controls) contained 1% ethanol. Points plotted for ATP represent averages for three determinations; control ATP = 3.00 ± 0.19 mmol per kg of cell water; average S.E.M. for ATP data = 3%. Control points plotted for respiration are averages of 20 to 27 determinations; all others are averages of duplicate trials. Control value for wild type, 72.0 ± 2.7 μ l of O_2 per mg dry weight per hour; overall control for *poky f*, 129 ± 8 μ l of O_2 per mg dry weight per hour. (These values are lower than the values elsewhere (Figs. 1, 2, and 3) because of a time-dependent decline in respiration following the transfer of cells from growth medium to buffer (12).)

fluoromethoxyphenylhydrazone had multiple effects, uncoupling (stimulating respiration) at low concentrations but inhibiting both respiration and fermentation at higher concentrations. For this reason, the use of CCCP¹ to measure Site I phosphorylation in the present study required careful control experiments to identify the appropriate concentration range.

The effects of CCCP on respiration and on cellular ATP levels in wild type *Neurospora* are shown in Fig. 8A. CCCP began to stimulate respiration at 10^{-7} M, with peak stimulation (about 40%) at 10^{-6} M; higher concentrations caused progressive inhibition. Cellular ATP (measured after 30 s) began to fall at 10^{-7} M CCCP and reached a constant low level at 10^{-5} M. *Poky f* (Fig. 8B) showed respiratory uncoupling and inhibition over

¹ The abbreviation used is: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

roughly the same range of concentrations as did wild type *Neurospora*, but with three qualifications: CCCP did not stimulate respiration via the alternate oxidase (cyanide-resistant respiration; Fig. 8B, middle curve) but did stimulate total respiration (Fig. 8B, upper curve) and respiration via the cytochrome chain (Fig. 8B, lower curve); CCCP at higher concentrations inhibited respiration in all three cases, but inhibition of the cytochrome chain in *poky f* was less steep than in the wild type, with equivalent effects being shifted up the concentration scale by 0.5- to 1.0-log unit. The respiration results in themselves do not give a clear picture concerning Site I in *poky f*. The stimulation of total respiration by CCCP is most easily explained by the presence of Site I as a rate-limiting step affecting both branches of the respiratory system. Otherwise, one would expect the increased electron flow through the cytochrome chain (as a result of uncoupling at Sites II and III) to proceed at the expense of the electron flow through the alternate oxidase, with no change in total respiration. But if Site I is present, one would also expect CCCP to stimulate the alternate oxidase when measured separately (as cyanide-resistant respiration) and no such stimulation was observed. Evidently the mechanisms controlling the electron flow in the branched respiratory system of *poky f* are quite complex and further work will be required to clarify them.

The results shown in Fig. 8 do serve their main purpose, however, of identifying a range of CCCP concentrations (near 10^{-5} M) at which uncoupling is complete but at which substantial respiration still remains. The effect of the critical concentration of CCCP on ATP in *poky f* is shown in Fig. 9. CCCP (10^{-5} M) (middle curve) produced essentially the same sudden decline of ATP as had 1 mM cyanide, to 40% of the control value in about 15 s, but followed by a significantly smaller recovery, to about 60%. (In two other experiments, 3×10^{-5} M and 5×10^{-5} M CCCP gave nearly identical results, with ATP recovering to 65% and 62%, respectively. The slightly lower stabilized value of ATP (52%; Fig. 9) with cyanide plus CCCP can be attributed to the inhibition of respiration by this pair of compounds (Fig. 8B).)

From a comparison of the cyanide curve and the CCCP curve, one can calculate that as much as 22% ($0.22 = (77 - 60)/77$) of the steady state ATP production in the presence of cyanide might come from Site I phosphorylation. However, this estimate depends upon the assumption that CCCP acts specifically to prevent oxidative phosphorylation; and in particular, the estimate will be too high if CCCP simultaneously accelerates ATP breakdown. Two pathways for breakdown are of special concern: in the mitochondria, because the ATPase activity of isolated mitochondria is well known to be stimulated by uncouplers, and at the plasma membrane, because CCCP might be expected to make the membrane permeable to hydrogen ions (23, 24), thereby short circuiting the membrane potential and increasing the energy drain required to accomplish net transport across the membrane. Neither of these possibilities can be evaluated directly in intact cells, so that it would be fair at this stage to regard the figure of 22% only as setting an upper limit on the amount of Site I phosphorylation.

Acetate-grown Cells

We next considered whether it might be possible to amplify the contribution of Site I by changing the experimental conditions. The results described up to this point were obtained with sucrose-grown cells, in which there is presumably a substantial flow of carbon through the glycolytic pathway. By contrast, when *Neurospora* is grown on acetate, glycolysis is dramatically

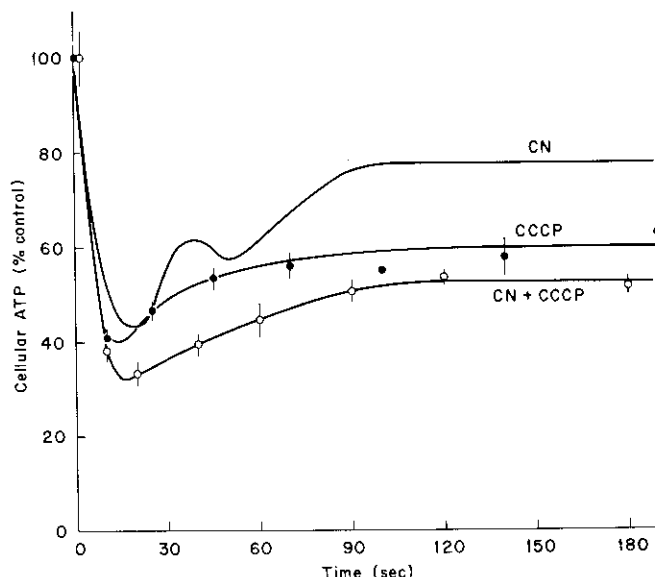


FIG. 9. Effect of CCCP on cellular ATP in *poky f*. Methods as in Figs. 5 and 8. Middle curve run with 10^{-5} M CCCP, lower curve with 5×10^{-5} M CCCP plus 10^{-3} M cyanide. Upper curve redrawn from Fig. 5 for comparison. Control ATP values: upper curve, 3.45 ± 0.16 mmol per kg of cell water; middle curve, 4.04 ± 0.07 mmol per kg of cell water; lower curve, 2.43 ± 0.14 mmol per kg of cell water.

reduced, enzymes of the glyoxylate cycle are induced, and energy for growth comes predominantly from the feeding of acetate directly into the Krebs cycle (25-30). If Site I is present, it should play a much more important role during growth on acetate. In particular, because 2 molecules of ATP are required to activate each acetate, and because only one ATP per acetate can be generated by substrate-level phosphorylation during the oxidation of α -ketoglutarate, net synthesis of ATP in the presence of cyanide should depend almost entirely on Site I, and should be extremely sensitive to the uncoupler.

One practical matter had to be explored before the ATP experiment could be carried out. Although wild type *Neurospora* grows quite well on acetate (with a doubling time of 3.5 hours at 25°, compared with 2.5 hours on sucrose), one might expect cytochrome-deficient mutants such as *poky* to be severely restricted. Indeed, the exponential growth rate of *poky* (lacking the nuclear suppressor *f*) was slowed almost 3-fold on acetate as compared with sucrose (doubling times of 14.6 and 5.3 hours, respectively). Growth of *poky f* was less affected, however, with doubling times of 6.8 hours and 4.5 hours on acetate and sucrose, respectively. Respiration in acetate-grown cells of *poky f* (219 μ l per mg dry weight per hour) was slightly faster than that in sucrose-grown cells (198 μ l per mg dry weight per hour), and the fraction going through the cytochrome chain (salicyl hydroxamic acid-resistant) accounted for 51% of the total instead of 28%. The average ATP level in acetate-grown *poky f*, transferred to the standard dimethylglutarate buffer with acetate added, was 2.1 ± 0.1 mmol per kg of cell water, 62% of the value seen in sucrose-grown cells. Over-all, the results of these preliminary experiments indicated that it was possible to obtain acetate-grown cells of *poky f* in reasonable physiological condition, so that the ATP experiment was feasible.

The effect of 1 mM cyanide on ATP in acetate-grown *poky f* is shown in Fig. 10 (upper curve). Upon the addition of cyanide, cellular ATP dropped sharply, with an exponential time constant of approximately 7 s, twice as fast as was observed with sucrose-

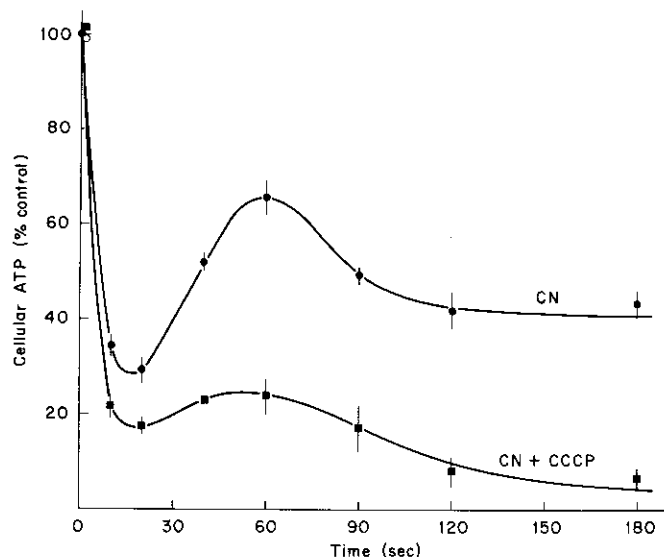


FIG. 10. Effect of cyanide, with and without CCCP, on cellular ATP in acetate-grown *poky* f. Methods generally as in Figs. 5 and 8, except that Vogel's medium (for growth) and the standard dimethylglutarate buffer (for ATP measurements) contained 1.13% sodium acetate instead of sucrose or glucose. Cyanide used at 1 mM; CCCP used at 5×10^{-5} M. Each point is the average of three determinations; vertical bars, ± 1 S.E. Control ATP values: cyanide curve, 1.90 ± 0.10 mmol per kg of cell water; cyanide + CCCP curve, 2.38 ± 0.02 mmol per kg of cell water.

grown cells (see Figs. 5 and 7). ATP reached a minimum of 28% of the control value in 15 s, subsequently rose to 66% at 1 min, and then declined again, stabilizing at 43% after 2 to 3 min. Three aspects of the curve are of interest. The apparent rate of ATP turnover in acetate-grown cells can be estimated, given a reasonable asymptote, say 20%, for the decay process, to be $(1.9 - 0.38)/7 = 0.22$ mmol per s, which is the same value as that found for sucrose-grown *poky* f. The large transient rise of ATP started off in the same manner as the recovery seen with sucrose-grown cells, but developed into a much larger and slower oscillation, with a period of nearly 90 s, instead of 30 s as seen in Fig. 5. Finally, the stable level of ATP in cyanide was substantial (43%), though lower than the corresponding value in sucrose-grown cells (77%; Fig. 6), and was totally sensitive to CCCP (Fig. 10, lower curve). The fact that a significant amount of ATP was maintained when respiration was restricted to the alternate oxidase supports the presence of a functional Site 1. The further reduction of ATP to 5% or less in the presence of CCCP is of course consistent with the same notion, although one must keep in mind, as previously, the possibility that CCCP sensitivity reflects as much a stimulation of ATP breakdown as an inhibition of synthesis.

Over-all, the results presented in this section and the preceding one agree at least qualitatively with the idea that during growth on sucrose, a significant fraction of ATP obtained via the alternate oxidase (78% or more) comes from substrate-level phosphorylation. During growth on acetate, though, there is a decline in glycolysis, and at the same time an increase in Krebs cycle enzymes (25–30), Site I phosphorylation (Fig. 10), and respiration through the cytochrome chain (see previous paragraph).

Changes in ADP and AMP

To investigate further the regulatory process operating on ATP levels during short term exposure to inhibitors, we have followed the time courses of ADP and AMP, as well as ATP, in the three

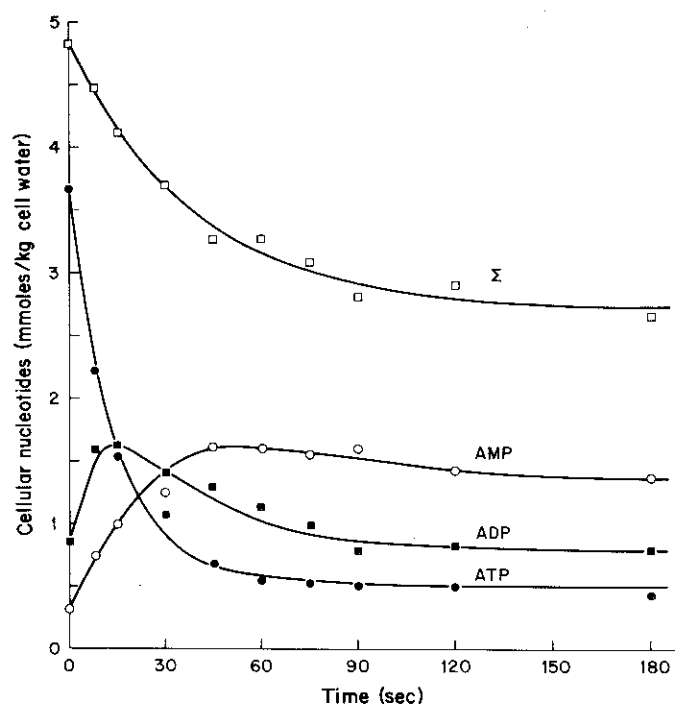


FIG. 11. Effect of cyanide + salicyl hydroxamic acid on adenine nucleotides in *poky* f. Methods as in Fig. 5; data from the same experiment as in Fig. 5. Curves for the sum (Σ) and for ATP were drawn according to the equation $y = A_1 + A_2 e^{-t/\tau}$, with the values below obtained by least squares (15). Curves for ADP and AMP were drawn by eye, constrained so that $ATP + ADP + AMP = \Sigma$. Average S.E.M. values for the data: ATP, 0.09; ADP, 0.11; AMP, 0.18.

	A_1	A_2	τ
	mmol per kg of cell water		s
ATP	0.52 ± 0.04	3.13 ± 0.07	14.1 ± 1.0
Σ	2.67 ± 0.08	2.14 ± 0.09	40.1 ± 5.7

experiments presented in Figs. 5, 6, and 10 for the *poky* f strain. The simplest result to analyze is that for treatment with cyanide + salicyl hydroxamic acid, shown in Fig. 11. The curves for all three nucleotides were practically identical with those obtained previously during the cyanide blockade of wild type *Neurospora* (12), except for the slower decay of ATP (already noted), with a resultant slower rise in ADP, and for the absence of any recovery of ATP. The three curves conform qualitatively to the expectation for the following system of reactions: $ADP + P_i \rightleftharpoons ATP$; $2ADP \rightleftharpoons ATP + AMP$; and $AMP \rightleftharpoons$ Adenosine + P_i . (Of these equations, only the second is a defined reaction, catalyzed by adenylate kinase; the others represent the sum of all of the processes leading to synthesis and degradation of ATP and AMP.) The characteristic shape of the ADP curve, rising sharply during the rapid phase of ATP decline and then falling, should be determined by a relatively rapid equilibrium at adenylate kinase. No feedback process is required to generate the experimental curves.

As expected from Fig. 6, treatment of *poky* f with cyanide alone yielded more complicated time courses for ADP and AMP (shown in Fig. 12). Both rose, reached peaks close to the time of the ATP minimum, and then declined in what appeared to be a two-phase process, synchronized with the minor oscillation of ATP. In the case of cyanide treatment of acetate-grown cells (Fig. 13), ADP started off at a high level, about 1.2 mmol per

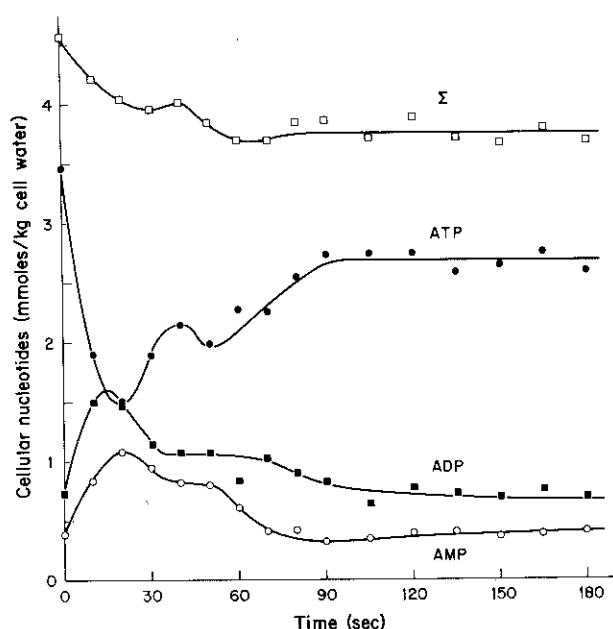


FIG. 12. Effect of cyanide on adenine nucleotides in *poky f*. Data from the same experiment as in Fig. 6. Curves were drawn by eye, but constrained so that $ATP + ADP + AMP = \Sigma$. Average S.E.M. values: ATP, 0.14; ADP, 0.12; AMP, 0.07.

kg of cell water, and stayed very near there through the entire interval of inhibition. AMP and ATP oscillated almost exactly 180° out of phase.

It is somewhat easier to analyze these results in tabular form. Table IV lists the nucleotide concentrations plotted in Figs. 11 to 13, as well as three additional functions: the apparent equilibrium constant for adenylate kinase ($K = ATP \cdot AMP / ADP^2$); the ratio $AMP:ATP$; and the reciprocal ATP concentration. From the values of ADP and also from the values of K in all three sets of data, ADP seems unlikely to be critically involved in the feedback process leading to ATP recovery. The equilibrium for adenylate kinase is established relatively rapidly (K values at 2 to 3 min are nearly equal to those at zero time); and that equilibrium returns ADP to its control level within 2 min, regardless of whether or not ATP shows recovery. Regulation by AMP alone also seems unlikely, because in the case of cyanide inhibition (Fig. 12), AMP returns to the control value in slightly more than 1 min, whereas recovery of ATP is completed only slowly (on the time scale of the growth experiments). Control by $1/ATP$ could conceivably mediate the recovery seen in the short term experiments, but not in the long term growth experiments, where ATP does eventually reach the control level (at depressed growth rates).

If the control is mediated by the adenine nucleotides, then the ratio $AMP:ATP$ is left as the only parameter which could produce both the short and long term recovery of ATP in *poky f*. This ratio, which amplifies small changes in AMP and ATP, stabilizes almost 40-fold elevated ($3.3:0.085$) when ATP recovery is prevented by the complete blockade of respiration. It stabilizes 6-fold higher than the control with cyanide inhibition of acetate-grown cells. Also, in cyanide-inhibited sucrose-grown cells, after declining very nearly to the control value ($0.12:0.11$) at 90 s, the ratio rises steadily with time, reaching $1.6\times$ by 3.5 min. Nevertheless, it should be apparent that elevation of the $AMP:ATP$ ratio by 1.6-fold represents a small variation in a putative control parameter and a survey of other possible control mechanisms would be worthwhile.

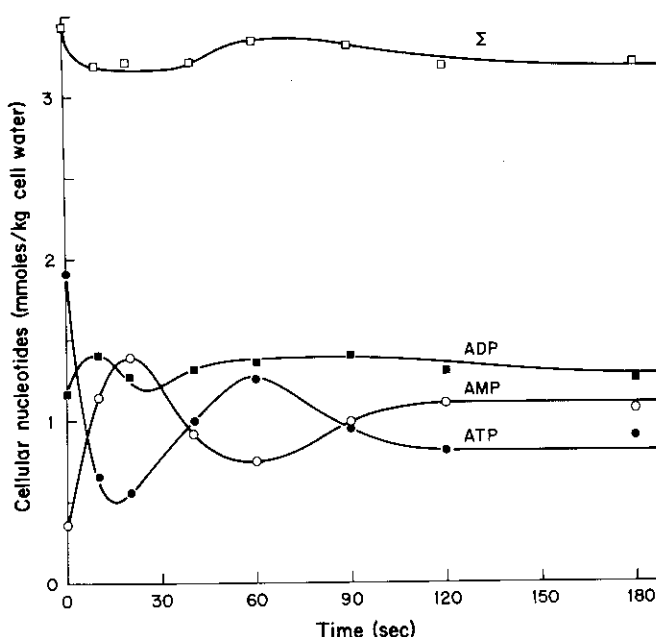


FIG. 13. Effect of cyanide on adenine nucleotides in acetate-grown *poky f*. Data from the same experiment as in Fig. 10. Curves were drawn by eye, but constrained so that $ATP + ADP + AMP = \Sigma$. Average S.E.M. for all points: 0.06.

TABLE IV

Various adenine nucleotide functions during inhibition of *poky f* by cyanide and cyanide plus salicyl hydroxamic acid

Listed values for the three nucleotides represent the data plotted in Figs. 11 to 13. K is the apparent equilibrium constant for adenylate kinase, $(ATP \cdot AMP) / (ADP)^2$. CN is cyanide and SHAM is salicyl hydroxamic acid.

Treatment	Time (sec)	ATP (nmoles/kg cell water)	ADP (nmoles/kg cell water)	AMP (nmoles/kg cell water)	K	$\frac{AMP}{ATP}$	$\frac{1}{ATP}$ (μM^{-1})
Sucrose-grown cells CN + SHAM FIG. 11	0	3.66	0.85	0.31	1.57	0.085	0.27
	15	1.52	1.61	0.95	0.57	0.64	0.66
	30	1.06	1.39	1.24	0.68	1.17	0.94
	45	0.67	1.29	1.29	0.52	1.92	1.49
	60	0.53	1.12	1.60	0.68	3.02	1.89
	90	0.49	0.77	1.53	1.26	3.12	2.04
	120	0.49	0.81	1.58	1.18	3.22	2.04
	180	0.43	0.78	1.42	1.00	3.30	2.32
Sucrose-grown cells CN alone FIG. 12	0	3.45	0.72	0.38	2.53	0.11	0.29
	10	1.89	1.50	0.82	0.69	0.43	0.53
	20	1.49	1.47	1.07	0.74	0.72	0.67
	40	2.13	1.06	0.82	1.55	0.38	0.47
	60	2.26	0.83	0.60	1.97	0.26	0.44
	90	2.72	0.81	0.32	1.33	0.12	0.37
	120	2.74	0.75	0.39	1.90	0.14	0.36
	180	2.59	0.68	0.40	2.24	0.15	0.39
Acetate-grown cells CN alone FIG. 13	0	1.90	1.17	0.36	0.50	0.19	0.53
	10	0.65	1.40	1.14	0.38	1.75	1.54
	20	0.56	1.26	1.38	0.49	2.46	1.79
	40	0.99	1.30	0.91	0.53	0.92	1.09
	60	1.25	1.35	0.74	0.51	0.59	0.80
	90	0.94	1.38	0.98	0.48	1.04	1.06
	120	0.80	1.29	1.09	0.52	1.36	1.25
	180	0.89	1.24	1.06	0.61	1.19	1.12

DISCUSSION

Physiological Role of Alternate Oxidase—Cyanide-resistant respiration has now been reported in numerous plant tissues (31–33) and fungi (7, 34–36), and where it has been studied in detail, has been found to depend upon an alternate oxidase which branches from the normal respiratory chain at or just beyond the flavoprotein level (2, 5, 7, 31). Because the alternate oxidase occurs in such a wide variety of organisms and because studies with isolated mitochondria have shown that no oxidative phos-

phorylation is coupled directly to it (3, 4, 32, 33), there has been considerable speculation as to its physiological role. In one special case—the skunk cabbage spadix, which grows at extremely low temperatures—Bahr and Bonner (32) have calculated that respiration through the alternate oxidase may be important in heat production. In other plant and fungal cells, however, a more general role has been postulated in helping to reoxidize pyridine nucleotides and other metabolic intermediates and in stimulating ATP synthesis indirectly (3, 32).

The experiments described in this paper were undertaken to investigate the latter point and have revealed that indeed a small but significant amount of ATP synthesis (partly substrate-level phosphorylation, partly oxidative phosphorylation at Site I) does occur during respiration through the alternate oxidase in *Neurospora*. In *poky f*, both the indirect measurements of growth rate and direct measurements of cellular ATP have made it possible to calculate that the alternate oxidase is 18 to 21% as efficient as the defective *poky f* cytochrome chain (and about 13% as efficient as the wild type cytochrome chain) in generating ATP. In fact, enough ATP is produced to support slow growth in the complete absence of respiration through the cytochrome chain (*poky f*, in medium containing antimycin). Although all of the presently known mitochondrial mutants of *Neurospora* retain some residual cytochrome respiration (2, 4, 37), one would expect mutants completely lacking one or more components of the cytochrome chain to be viable.

Even though the alternate oxidase is useful in by-passing a blocked cytochrome system, it does so at the expense of net phosphorylating efficiency; and at least two regulatory mechanisms exist to ensure that, under any given circumstance, the cytochrome system is used preferentially. The first mechanism is at the level of synthesis. Wild type *Neurospora*, growing in the standard medium, contains virtually none of the alternate oxidase and only when the cytochrome chain is inhibited or altered genetically does synthesis begin (2–4, 7, 8). An additional mechanism then takes effect at the level of activity. As demonstrated both for plant mitochondria (32–33) and for *Neurospora* mitochondria (3), electron flow through the branched respiratory system is somehow partitioned such that the cytochrome chain is filled to capacity and the alternate oxidase functions only to accommodate the surplus. Both regulatory mechanisms are interesting in themselves and deserve further study.

Comparison of Cytochrome-deficient Mutants—Most of the work described in this paper, as well as much previous work (2, 3, 7), has been done with *poky* containing the nuclear-gene suppressor *f*, because the presence of *f* leads to faster growth and to more stable mitochondria. The results of Fig. 4 and Table II confirm the fact that the branched respiratory system is not unique to this particular strain, but occurs in other mutants as well: *poky* without *f*, *mi-3* (see also Ref. 5), and C117. Furthermore, they indicate that differences in growth rate among the various mutants can be accounted for quantitatively by differences in the amount of respiration through the cytochrome chain versus the amount through the alternate oxidase. This finding is of particular interest in attempts to understand the mechanism of the *f* suppressor. Mitchell and Mitchell (10) originally identified *f* by its effect on the growth rate of *poky* and reported, on the basis of hand-spectroscopic observations, that *f* had no marked effect on the cytochrome spectrum. It is now clear that *f* does lead to a small but significant increase in respiration through the cytochrome chain, which in turn is sufficient to account for the increased growth rate. In confirmation of the results of Mitchell and Mitchell (10) and Bertrand and Pittenger (38), *f*

is a specific suppressor of the *poky* class of mutations, and has little effect on either respiration or growth rate in *mi-3* (Fig. 4, Table IV).

Sites of Synthesis of ATP—We now turn from general questions concerning the efficiency of the *Neurospora* respiratory system to more specific questions about ATP synthesis. The results presented in this paper suggest that, in addition to oxidative phosphorylation at Sites II and III linked to the cytochrome chain, *Neurospora* carries out oxidative phosphorylation at Site I and substrate-level phosphorylation, which are linked to both branches of the respiratory system. Because of uncertainties in interpreting the results with CCCP (discussed earlier), it is not possible to calculate the exact contributions of these two processes. In sucrose-grown *poky f*, Site I may account for as much as 22% of ATP synthesis during respiration through the alternate oxidase, with substrate-level phosphorylation accounting for the remainder. In acetate-grown cells, which have enhanced levels of Krebs cycle enzymes and little glycolysis (25–30), there is a correspondingly greater contribution of Site I and smaller contribution of substrate level phosphorylation. The occurrence of Site I in intact cells is of special interest because previous work has shown it to be quite inefficient in mitochondria isolated from wild type *Neurospora* and undetectable in mitochondria isolated from *poky f* (3). Site I is generally labile in the fungi, however (39–42), and further work is needed to determine the conditions under which it functions best in *Neurospora*.

Feedback Control of ATP—One of the most striking observations made during the present experiments involves the response of cellular ATP in *poky f* to cyanide. In theory, the recovery of ATP from the initial inhibited level (43%) to 77% of the control value, over a 3-min period, could reflect either a feedback stimulation of ATP synthesis or a feedback inhibition of ATP breakdown. For two reasons, the latter alternative seems more likely. In longer term experiments with *poky f*, the growth rate (and therefore the over-all rate constant for ATP breakdown) was clearly regulated to keep the cellular ATP level high (2.5 to 3.0 mmol per kg of cell water). It seems reasonable to believe that the short term response of ATP to inhibitors is a reflection of the same regulatory process. Furthermore, it is difficult to imagine a stimulation of ATP synthesis, in the presence of cyanide, that would be large enough to account for the observed recovery of cellular ATP. Parallel measurements showed that the over-all rate of respiration did not increase in the presence of cyanide, but that respiration merely shifted to the alternate oxidase. One would therefore have to invoke nearly a 2-fold increase in the efficiency of all phosphorylations linked to the alternate oxidase, and this seems unlikely.

Admittedly, however, feedback control of ATP breakdown also poses problems, because numerous enzymes and substrates are involved in ATP-using reactions within the cell. One would want to begin by examining reactions which normally consume a large fraction of total cellular ATP, e.g. nucleic acid or protein synthesis or the electrogenic ion pump which supports the resting membrane potential of *Neurospora* (18). (The latter possibility is an intriguing one. The electrogenic pump has been calculated to consume more than 25% of the total energy produced by respiring wild type cells (18). Furthermore, because the membrane potential underlies the uptake of glucose (43) and possibly other substrates in *Neurospora*, the inhibition of the electrogenic pump could well result in a general slowing down of metabolism as intracellular substrate concentrations decreased and could not be replenished.) The signal for inhibition of any of these ATP-

using reactions might be a change in the AMP:ATP ratio, as discussed in the last section under "Results." Alternatively, it might be a change in cyclic nucleotide concentrations. In this regard, Sturani *et al.* (44) have described the inhibition of total rRNA synthesis in *Neurospora* during the transition from growth on glucose to growth on glycerol. The response resembles that observed in stringent strains of bacteria which are deprived of nitrogen, sulfur, or carbon, except that guanosine tetraphosphate (which appears to mediate the response in bacteria) was not found in *Neurospora* (45). However, since the growth of other eukaryotic cells is known to be influenced by cyclic nucleotides, since the adenylate cyclase system has been identified in *Neurospora* (46-49), and since Chance *et al.* (50) showed sizeable effects of adenosine 3':5'-cyclic monophosphate on oscillations of NADH in a cell-free extract of *Saccharomyces carlsbergensis*, a search for variations of cyclic AMP in relation to the feedback control of growth in *poky f* might be fruitful.

Whatever the mechanism of feedback control, its net result is to keep cellular ATP in *poky f* high (in fact, slightly above the wild type level), a phenomenon also observed in the yeast *Rhodotorula gracilis* (51). Previous hypotheses concerning the mechanism of induction of the glyoxylate shunt (27) and, more recently, the mechanism of induction of the alternate oxidase (8, 20) have made use of data from *poky*, on the assumption that cellular ATP in *poky* is low. We now see that this assumption is invalid, unless one assumes in addition that the ATP of significance exists in a subcellular compartment where the concentration is lower in *poky* than in the wild type. Although morphologically distinct subcellular compartments certainly exist in *Neurospora*, making it unlikely that ATP (or indeed any other compound) is uniformly distributed throughout the cell water, available biochemical and physiological data indicate that the exchange of ATP among such compartments must be extremely rapid. When the wild type strain or *poky f* is exposed to cyanide plus salicyl hydroxamic acid, cellular ATP falls to 10 to 15% of its initial value with a single exponential time constant. Furthermore, the fact that at least one ATP-using reaction, the membrane potential, falls in close parallel with total ATP means that essentially all of the cellular ATP, wherever it is located initially, can be made available without significant delay in moving from one "compartment" to another.

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